Colocalization of Cystatin M/E and Cathepsin V in Lamellar Granules and Corneodesmosomes Suggests a Functional Role in Epidermal Differentiation

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Cystatin M/E is a cysteine protease inhibitor with two distinct binding sites for papain-like cysteine proteases (family C1) and the asparaginyl endopeptidase (AEP) legumain of family C13. We have previously demonstrated that deficiency of cystatin M/E in mice causes ichthyosiform skin changes and barrier disruption, which could be caused by unrestrained AEP activity. Recently, we provided biochemical evidence that human cathepsin V (CTSV) and cathepsin L (CTSL) are additional biological targets for human cystatin M/E. To address the possible role of these three proteases and their inhibitor in epidermal differentiation, we investigated the localization of these proteins in normal human skin. Whereas CTSL and AEP were broadly expressed in epithelial cells of the skin, we found a specific colocalization of cystatin M/E and CTSV in the stratum granulosum and in the root sheets of the hair follicle, using immunofluorescence microscopy. Immunoelectron microscopy revealed that cystatin M/E and CTSV are separately transported within the lamellar granules. Cystatin M/E was also found in the extracellular space in the stratum corneum associated with corneodesmosomes, where it was closely associated with CTSV. Based on the striking stratum-specific colocalization of cystatin M/E and CTSV, we propose that these molecules could have an important role in epidermal differentiation and desquamation.

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INTRODUCTION

Cystatin M/E is a cysteine protease inhibitor whose expression is largely confined to cutaneous epithelia. In human skin, it is expressed in the sweat glands, hair follicles, and stratum granulosum (SG) of the epidermis. In addition to its function as a cysteine protease inhibitor, cystatin M/E could also serve as a target for crosslinking by transglutaminases (TGase) (Zeeuwen *et al.*, 2001). We have previously reported that a null mutation in the mouse *cystatin M/E* gene (*Cst6*) causes the murine *ichq* phenotype, which is characterized by neonatal lethality and abnormalities in cornification and desquamation, demonstrating an essential role for cystatin M/E in the final stages of epidermal differentiation (Zeeuwen *et al.*, 2002). We have shown that absence of cystatin M/E and appearance of asparaginyl endopeptidase (AEP) activity colocalizes with the observed pathology in *ichq* mice, that is, excessive cornification in hair follicles and epidermis. Furthermore, these investigations have revealed that AEP could indirectly mediate the activation of TGase 3 zymogen, via lysosomal cysteine proteases *in vitro* (Zeeuwen *et al.*, 2004). A biochemical study from another group has indicated that cystatin M/E *in vitro* binds to AEP with high affinity (Alvarez-Fernandez *et al.*, 1999), which was an unexpected finding, as the cystatins were primarily regarded as inhibitors of the papain-like cysteine proteases in the unrelated family C1 (Abrahamson *et al.*, 2003). Although cystatin M/E contains a predicted binding site for papain-like cysteine proteases, no high-affinity binding for any member of this family has been published so far. Recently, we have shown that cystatin M/E is a high-affinity inhibitor of cathepsin V (CTSV) and cathepsin L (CTSL) by a reactive site that is distinct from the AEP-binding site (Cheng *et al.*, 2006). On the basis of the analogous sites in cystatin C (Alvarez-Fernandez *et al.*, 1999), we used site-directed mutagenesis to identify the binding sites of these proteases in human cystatin M/E, and we found that AEP and papain-like cysteine proteases are inhibited by two distinct non-overlapping sites. In addition, we showed that human CTSL is the elusive enzyme that is able to process and activate human TGase 3 (Cheng *et al.*, 2006). Altogether, these data strongly imply an important role for cystatin M/E and its protease targets in the differentiation process of human epidermis.

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Abbreviations: AEP, asparaginyl endopeptidase; CTSL, cathepsin L; CTSV, cathepsin V; KLK, kallikrein; LG, lamellar granule; SC, stratum corneum; SG, stratum granulosum; TGase, transglutaminase

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Desquamation is the final event in terminal differentiation of the epidermis, which occurs by the action of proteolytic enzymes. During desquamation, breakdown of lipids in the intercellular spaces and loss of residual intercellular desmosomal connections leads to cell shedding of the dead corneocytes from the stratum corneum (SC) surface (Elias, 2005). A number of studies have shown the involvement of different epidermal proteases in the progressive degradation of the desmosomal proteins desmocollin-1, desmoglein-1, plakoglobin, and corneodesmosin (Lundstrom and Egelrud, 1990; Suzuki *et al.*, 1996; Simon *et al.*, 2001; Caubet *et al.*, 2004). A convincing role in desquamation has been demonstrated for at least two epidermis-specific serine proteases, the SC chemotryptic and SC tryptic enzymes (currently known as kallikreins KLK7 and KLK5), which are able to degrade corneodesmosome proteins (Hansson *et al.*, 1994; Brattsand and Egelrud, 1999; Ekholm *et al.*, 2000; Caubet *et al.*, 2004). KLK7 and KLK5 are highly expressed in the SG, and are present in the intercellular spaces of the SC (Sondell *et al.*, 1995). Both these enzymes are most active at neutral to alkaline pH, but also showed some activity at a more acidic pH. In addition to these serine proteases, both cysteine and aspartic protease activities with acidic pH optima have been found in the SC (Horikoshi *et al.*, 1999; Watkinson, 1999). We have previously suggested that a new cysteine protease of late epidermal differentiation, stratum corneum thiol protease, could be a possible target for cystatin M/E (Zeeuwen *et al.*, 2001). Stratum corneum thiol protease appears to be a specific product of differentiated keratinocytes, and furthermore it is apparently not stored within these cells but is actively secreted (Watkinson, 1999). Because stratum corneum thiol protease is able to degrade desmocollin-1 in *in vitro* degradation experiments, an extracellular proteolytic function for this protease in mediating desquamation has been proposed. It was shown by others that ''stratum corneum thiol protease'' activity was also able to hydrolyze corneodesmosin, and finally it appears that this so-called stratum corneum thiol protease was identical to CTSL2 (Bernard *et al.*, 2003), also known as CTSV (Adachi *et al.*, 1998). Our recent data on CTSV and CTSL as additional biological targets for cystatin M/E suggest that these enzymes could possibly play an important regulatory role in epidermal barrier formation and desquamation.

In this study, we sought to address the possible role of cystatin M/E and its physiological target proteases in epidermal differentiation and desquamation. We have examined the localization and compartmentalization of cystatin M/E, CTSV, CTSL, and AEP in normal human skin by immunofluorescence microscopy and immunoelectron microscopy. We found that cystatin M/E and CTSV are separately transported within lamellar granules (LGs). Both proteins are secreted in the extracellular space where they associate with corneodesmosomes, suggesting a regulatory role for cystatin M/E in the desquamation process.

RESULTS

We have recently shown that cystatin M/E is an inhibitor of CTSV and CTSL by a reactive site that is distinct from the AEP-binding site. For a comprehensive biochemical and kinetic analysis of the interaction between cystatin M/E and its target proteases, see Cheng *et al.* (2006).

In order to examine the localization of cystatin M/E and its physiological target proteases CTSV, CTSL, and AEP in normal human skin, immunofluorescence microscopy was performed (Figures 1–3). A strong and consistent staining of cystatin M/E was found in the SG (Figure 1a) and in the inner half of the root sheet of the hair follicle (Figure 1d). Staining of the SC was present but often discontinuous (Figure 1a). Furthermore, cystatin M/E was found in the secretory coil epithelium of the eccrine sweat glands (Figure 1g), but not in the ductal portion. In the sebaceous glands, only the inner mature cells were positive (Figure 1j). Cystatin M/E specifically colocalizes with CTSV in the most superficial granular layer, whereas colocalization in the SC shows a patchy distribution (Figure 1c and m). Double immunostaining revealed that cystatin M/E was expressed earlier during epidermal differentiation than CTSV (Figure 1c and m). At higher magnifications, immunofluorescent staining for cystatin M/E and CTSV shows a typical granular pattern (Figure 1n and o). Cystatin M/E and CTSV also colocalize in the most inner layer of the root sheet of the hair follicle (Figure 1f), and in a few cells of the sebaceous glands (Figure 1l). CTSL and AEP are both broadly expressed throughout the epidermis and its appendages (Figures 2 and 3). At higher magnifications, immunofluorescent staining for CTSL and AEP shows a diffuse staining pattern (not shown). Colocalization of cystatin M/E with CTSL was found at the cellular level in the SG, in the inner half of the hair follicles root sheet, in the secretory coil epithelium of the eccrine sweat glands, and in the inner mature cells of the sebaceous glands (Figure 2c, f, i, and l). We could not detect CTSL expression in the SC (Figure 2c). Furthermore, staining for cystatin M/E overlapped with that for AEP in the SG and the SC, in the inner half of the root sheet of the hair follicle, in the secretory coil epithelium of the eccrine sweat glands, and in the inner mature cells of the sebaceous glands (Figure 3c, f, i, and l).

As shown above, using immunofluorescence microscopy, we could demonstrate co-expression of cystatin M/E and its presumed targets at the cellular level, in the most superficial granular cells of the epidermis. In order to define the expression of cystatin M/E and its target proteases at the subcellular level, immunoelectron microscopy was performed. We used two different methods that we have recently described: post-embedding and cryo-ultramicrotomy electron microscopy (Ishida-Yamamoto *et al.*, 2004). In the first method, LG appeared as isolated granules as in conventional transmission electron microscopy. In contrast, the cryo-ultramicrotomy method showed different images of LG, namely branched tubules with a partial internal lamellar structure. Using post-embedding immunoelectron microscopy, we could show that cystatin M/E and CTSV gold labels are found in LG within the cells of the SG (Figures 4a and 5b), and in the extracellular spaces of the SC where both molecules are associated with desmosomes (Figures 4b–d and 5c and d). We used cryo-ultramicrotomy electron microscopy to study the coexpression of cystatin M/E and CTSV. It was

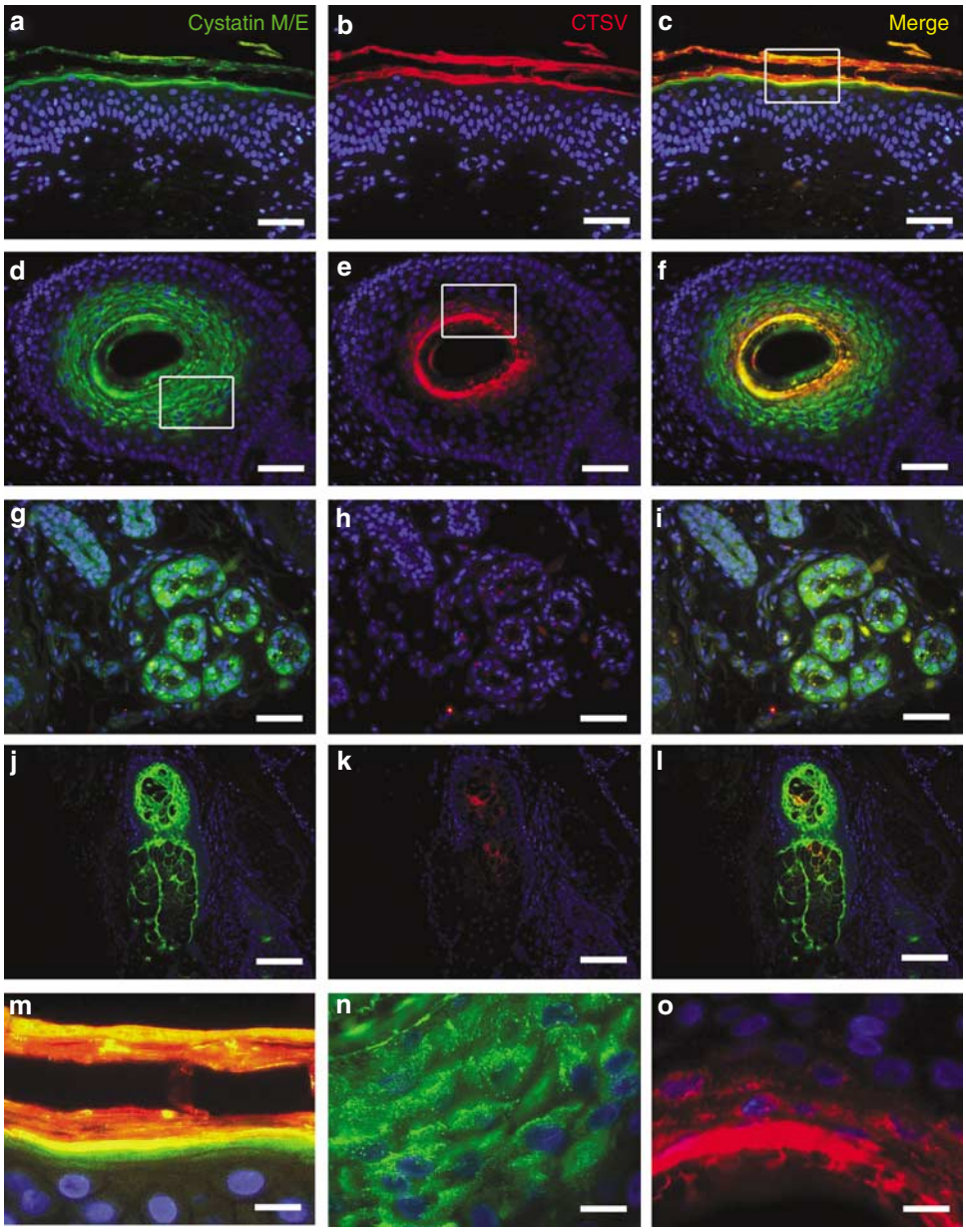

Figure 1. Colocalization of cystatin M/E and CTSV in human normal skin. Immunofluorescence staining of (green color; **a**, **d**, **g**, **j**) cystatin M/E and (red color; **b**, **e**, **h**, **k**) CTSV, and (yellow-orange merge color; **c**, **f**, **i**, **l**) double staining for both proteins in (**a**–**c**) the epidermis, (**d**–**f**) the hair follicle, (**g**–**i**) the sweat glands, and (**j**–**l**) the sebaceous glands. The rectangular area marked in (**c**) is shown enlarged in (**m**). Note that the SC is detached from the SG. The rectangular areas marked in (**d** and **e**) are shown enlarged in (**n** and **o**). DNA staining by 4′,6-diamidine-2′-phenylindole dihydrochloride is colored in blue. Bar= (**a**–**l**) 50 μm; (**j**–**l**) 100 μm; and (**m**–**o**) 12.5 μm.

found that both proteins are aggregated but not mixed with each other in the LG of the cells of the SG (Figure 6a and b). In the extracellular spaces of the SC, cystatin M/E was colocalized with CTSV, and both proteins are found to be closely associated with desmosomes (Figure 6c and d). Furthermore, post-embedding immunoelectron microscopy revealed that AEP and CTSL are not LG proteins. Labeled proteins were found in the cytoplasm of SG cells (Figures 7 and 8), where some of the labeling was associated with keratin filaments. No expression of AEP and CTSL was found in the extracellular spaces of the SC.

DISCUSSION

In this study, we analyzed the tissue localization and subcellular compartmentalization of cystatin M/E and its physiological target proteases in normal human skin. We showed for the first time that cystatin M/E and CTSV are separately transported within LG. Moreover, both proteins are secreted in the extracellular space where they associate with corneodesmosomes, suggesting a regulatory role for cystatin M/E and CTSV in epidermal desquamation.

Here, we showed by immunofluorescence microscopy that cystatin M/E appeared to be present together with AEP,

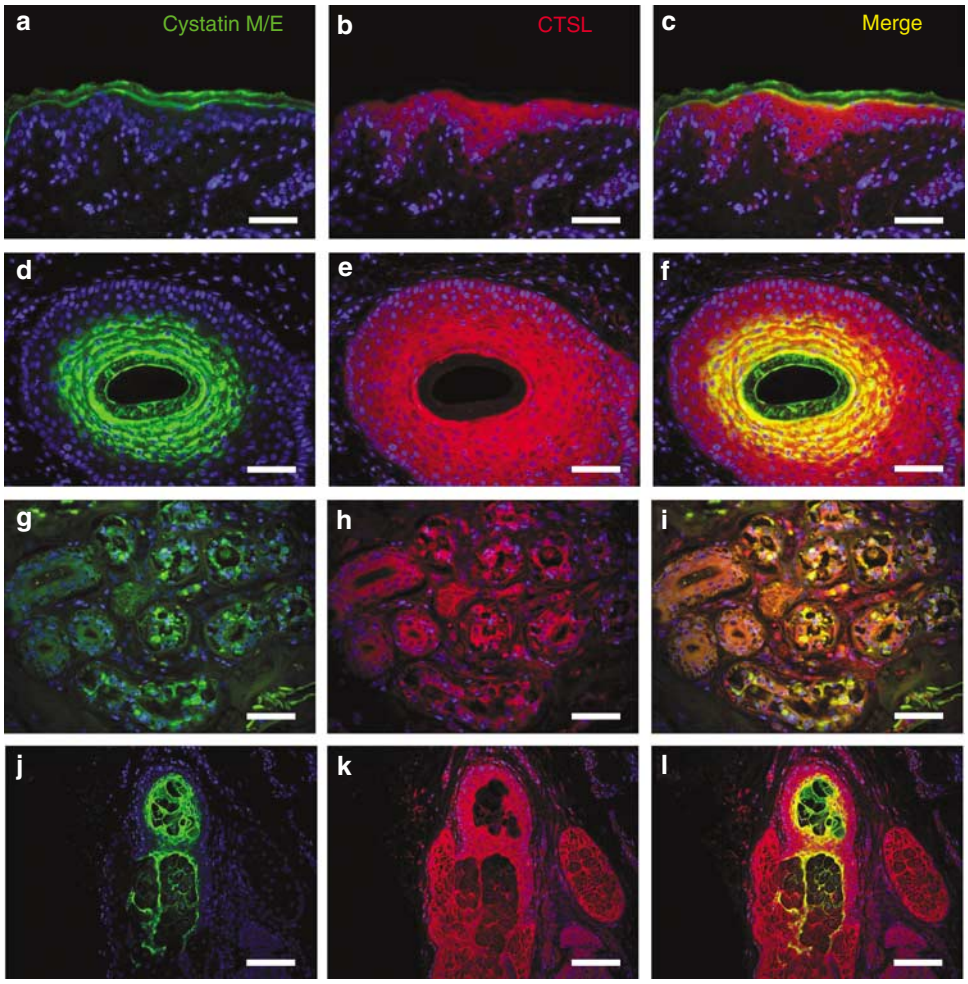

Figure 2. Colocalization of cystatin M/E and CTSL in human normal skin. Immunofluorescence staining of (green color; **a**, **d**, **g**, **j**) cystatin M/E and (red color; **b**, **e**, **h**, **k**) CTSL, and (yellow-orange merge color; **c**, **f**, **i**, **l**) double staining for both proteins in (**a**–**c**) the epidermis, (**d**–**f**) the hair follicle, (**g**–**i**) the sweat glands, and (**j**–**l**) the sebaceous glands. DNA staining by 4′,6-diamidine-2′-phenylindole dihydrochloride is colored in blue. Bar= (**a**–**l**) 50 μm and (**j**–**l**) 100 μm.

CTSL, and CTSV in some parts (mainly the most superficial cells) of the epidermis (Figures 1–3). The granular staining pattern in the cells that we found for cystatin M/E and CTSV suggested that both proteins are associated with LG. To evaluate this hypothesis, post-embedding immunoelectron microscopy was performed, and we indeed could confirm the localization of cystatin M/E and CTSV in LG, which appear as isolated oval-shaped granules (Figures 4 and 5). LG, or lamellar bodies, are secretory granules of keratinizing squamous epithelia and are thought to be essential in barrier formation and desquamation. It is widely accepted that LG originate from the Golgi apparatus, and that the assembly of these secretory granules begins at the trans most cisternae of the Golgi complex (Madison, 2003). Recent work on the *trans*-Golgi network, which is the highly tubulated sorting and delivery part of the Golgi apparatus, has revealed that transport to the plasma membrane is mediated by pleiomorphic tubulovesicular structures, rather than by vesicles (Mironov *et al.*, 2001). It was shown by high magnification electron microscopy that LG do not represent a uniform vesicular population, but are consistent with sections through a tubular network (Elias *et al.*, 1998), which suggest that keratinocyte LG are *trans*-Golgi network structures and not vesicles. Norlén has therefore proposed a new model for the delivery of the contents of LG to the plasma membrane (Norlen, 2001). This so-called ''membrane folding model'' implies that the *trans*-Golgi network and LG of the uppermost SG cells as well as the multilamellar lipid matrix of the intercellular space at the border zone between SG and SC could be representations of one and the same continuous membrane structure. Indeed, direct visualization of the skin barrier formation process using cryotransmission electron microscopy confirmed the presence of folded multilamellar continuous structures adjacent to apparent active sites of skin barrier formation (Norlen *et al.*, 2003).

Recently, it was shown that epidermal LG transport different cargoes (e.g., cathepsin D, glucosylceramides, corneodesmosin, and KLK7) as distinct aggregates, which were delivered to the apical region of granular keratinocytes (Ishida-Yamamoto *et al.*, 2004). Furthermore, it was shown

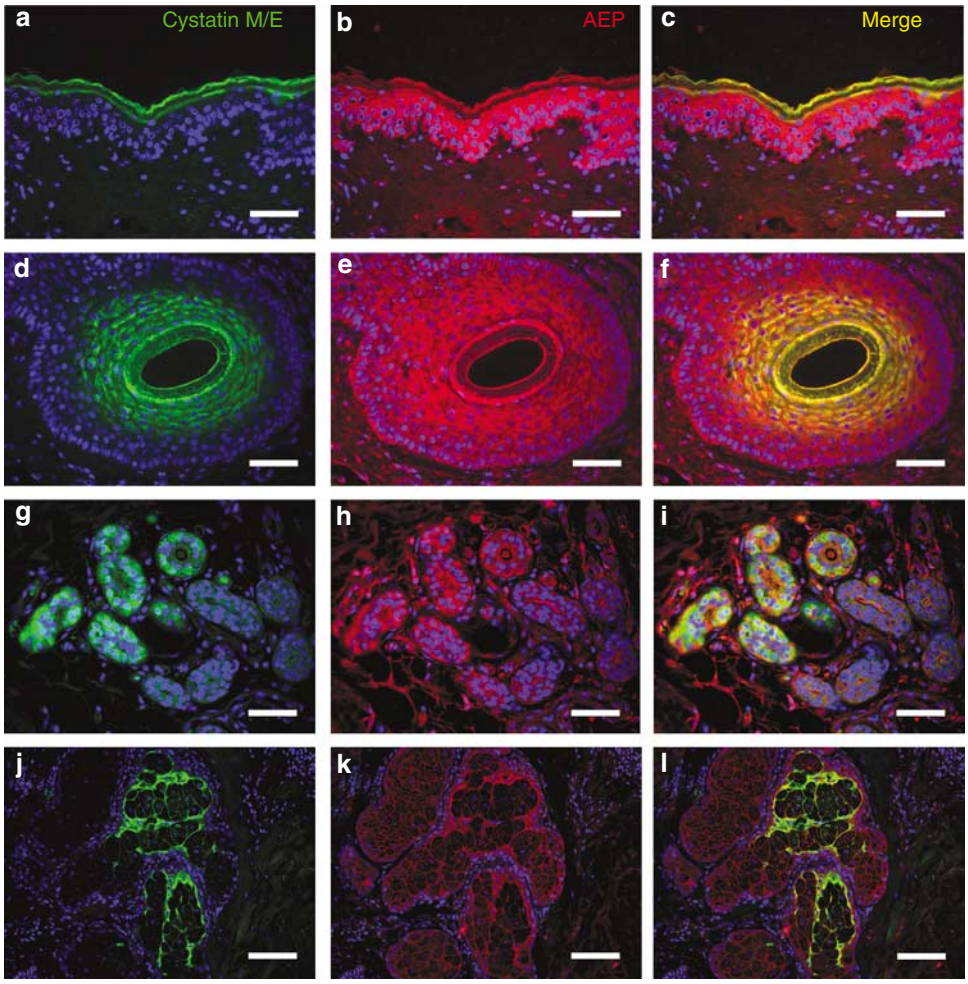

Figure 3. Colocalization of cystatin M/E and AEP in human normal skin. Immunofluorescence staining of (green color; **a**, **d**, **g**, **j**) cystatin M/E and (red color; **b**, **e**, **h**, **k**) AEP, and (yellow-orange merge color; **c**, **f**, **i**, **l**) double staining for both proteins in (**a**–**c**) the epidermis, (**d**–**f**) the hair follicle, (**g**–**i**) the sweat glands, and (**j**–**l**) the sebaceous glands. DNA staining by 4′,6-diamidine-2′-phenylindole dihydrochloride is colored in blue. Bar= (**a**–**l**) 50 μm and (**j**–**l**) 100 μm.

that the serine protease inhibitor LEKT1 (lympho-epithelial kazal type inhibitor) is localized in LG, separated from its epidermis-specific target proteases KLK7 and KLK5, and is secreted in the extracellular spaces of the superficial SG (Ishida-Yamamoto *et al.*, 2005). LEKT1 is encoded by the *SPINK5* gene, which is the causative gene of the severe ichthyotic skin disorder called Netherton syndrome (Chavanas *et al.*, 2000). Recent studies have reported that defective inhibitory regulation of epidermal serine protease activity owing to LEKT1 deficiency results in the early degradation of desmosomal proteins, possibly causing the skin pathology in Netherton syndrome (Caubet *et al.*, 2004; Ishida-Yamamoto *et al.*, 2005). We have observed a similar distribution for cystatin M/E and CTSV in LG using the cryo-ultramicrotomy method. Cystatin M/E and CTSV are both localized in the LG, however, double staining revealed that these proteins are localized separately within the presumed *trans*-Golgi LG network (Figure 6). Moreover, cystatin M/E and CTSV are detected in the extracellular spaces of the upper SC layers where both proteins are associated with desmosomes (Figures 4–6), suggesting that these molecules could have an important role in desquamation. Because an acidic pH dominates in the outer SC layers (Elias, 2004), it is conceivable that CTSV activity in these layers fulfills a significant function in the desquamation process (e.g., degradation of desmosomal constituents), as opposed to serine proteases which act in the neutral pH environment of the lower SC layers. One could speculate that separate transport of cystatin M/E and CTSV by LG may prevent interaction within the cells. Altogether, we propose a regulatory role for cystatin M/E in desquamation by controlling the CTSV activity in the SC (Figure 9).

In contrast with cystatin M/E and CTSV, the staining pattern for AEP and CTSL that we observed using fluoresence microscopy was diffuse and was not associated with granular structures. Immunoelectron microscopy showed that AEP and CTSL are not located within LG, but are found in the cytoplasm of the SG cell (Figures 7 and 8). As both AEP and CTSL are considered to be endosomal/lysosomal proteins, the cytosolic localization of both molecules in SG keratinocytes

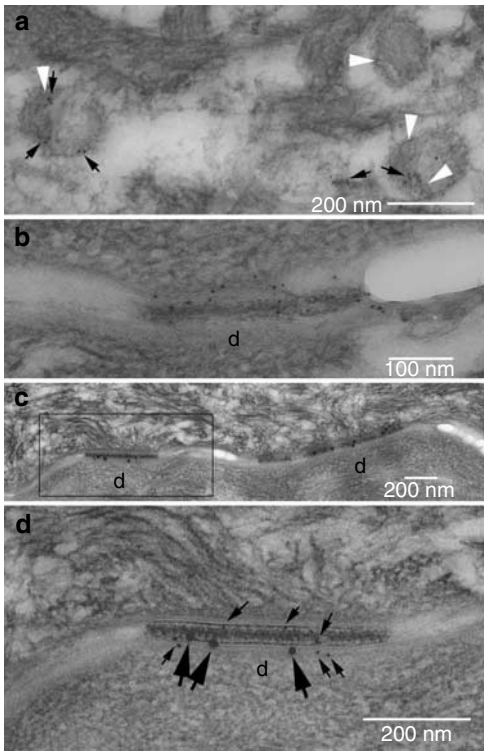

Figure 4. Cystatin M/E is an LG protein and is associated with desmosomes after secretion. Post-embedding immunoelectron microscopy using Lowicryl HM20 resin. (**a**) Cystatin M/E labels (5 nm gold, black arrows) are within the LGs. Note oval-shaped LGs with partial lamellar internal structures (white arrowheads). (**b**) In the SC, cystatin M/E labels are associated with desmosomes (d). (**c**, **d**) Double labeling of cystatin M/E (5 nm gold, smaller arrows) and desmoglein-1 (10 nm gold, larger arrows).

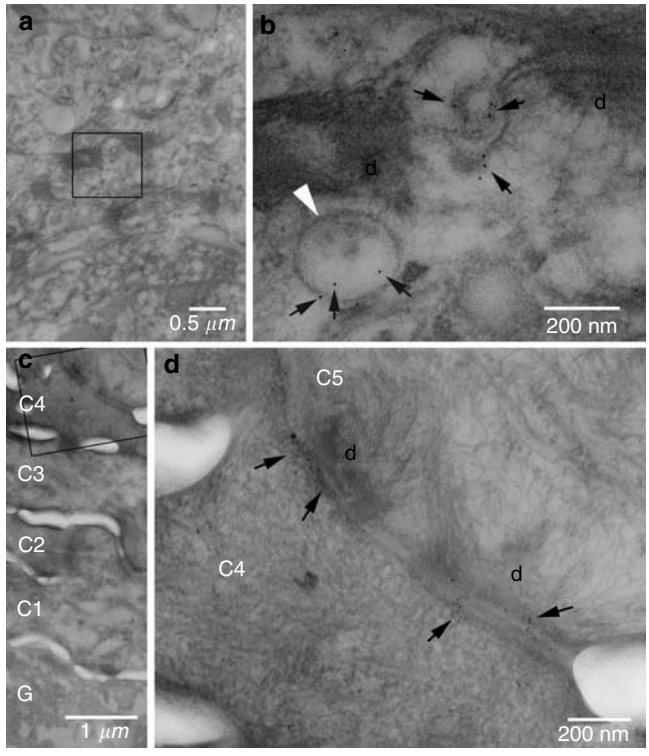

Figure 5. CTSV is an LG protein and is associated with desmosomes in the SC. Post-embedding immunoelectron microscopy using Lowicryl K11M resin. (**a**, **b**) SG of the epidermis where CTSV labels (5 nm gold, black arrows) are associated with LGs within the cells (white arrowhead) and those released into the extracellular spaces (upper right) between desmosomes (d). (**c**, **d**) SG (G) and the 1st (C1) to the 5th (C5) layer of the SC. CTSV labels are associated with desmosomes. The rectangular areas marked in (**a**) and (**c**) are shown at higher magnification in (**b**) and (**d**), respectively.

is at least remarkable. However, other studies have also shown that precursors of CTSL are found in the cytoplasm of epidermal keratinocytes (Kawada *et al.*, 1997), and in the cytoplasm of cortical thymic epithelial cells (Arudchelvan *et al.*, 2002). It has been coined that AEP, like CTSL, could be secreted from cells under some conditions, and may be active in the pericellular and intracellular environment (Barrett *et al.*, 2004). Furthermore, recent studies have reported increasing evidence that lysosomal proteases play important roles in physiological processes not solely restricted to lysosomes (Turk *et al.*, 2002). Protease activity and regulation outside lysosomes potentially contributes to the propagation of apoptosis, a process that is distinct from terminal differentiation of the epidermis, but nevertheless shares some molecular and cellular features (Lippens *et al.*, 2005). The signals for the release and activation of lysosomal proteases during cornification are unknown, and also the mechanism by which they degrade the cellular organelles.

In the epidermis, there is a balanced regulation of protease activity that, when disturbed, could lead to faulty cornification processes in the epidermis and upper part of the hair follicle (Zeeuwen, 2004). We have recently shown that human CTSL is the elusive enzyme that is able to process and activate human TGase 3 (Cheng *et al.*, 2006), an epidermis-specific enzyme responsible for the crosslinking of loricrin and small proline-rich proteins during the cornification process. The presence of TGase 3 in the cytoplasm of the cell is in accordance with the cytosolic localization of CTSL. At this point, we think that in normal healthy skin, post-translational proteolytic processing of TGase 3 is completed at the final stage of the granular cell layer when cell integrity is lost and proteases are released leading to progressively more TGase 3 enzyme activity. Despite the apparent loss of cell integrity, organization, and compartmentalization in the terminally differentiating keratinocyte, this must be a highly ordered and well-orchestrated process. We suppose that processing and activation of TGase 3 in terminally differentiating keratinocytes could be regulated by the inhibitory activity of cytoplasmic cystatin M/E against CTSL (Figure 9). AEP inhibition by cystatin M/E could have a regulatory role in CTSL activity as AEP is involved in CTSL processing (Maehr *et al.*, 2005). In addition, TGase 3 is also expressed in hair follicles, where it crosslinks soluble trichohyalin, a major structural protein in hair follicle differentiation (Tarcsa *et al.*, 1997). In this study, we show that cystatin M/E and its target proteases are all present in the root sheet of the hair follicle, which suggests that these molecules could play an important role in hair follicle morphogenesis. Moreover, both cystatin

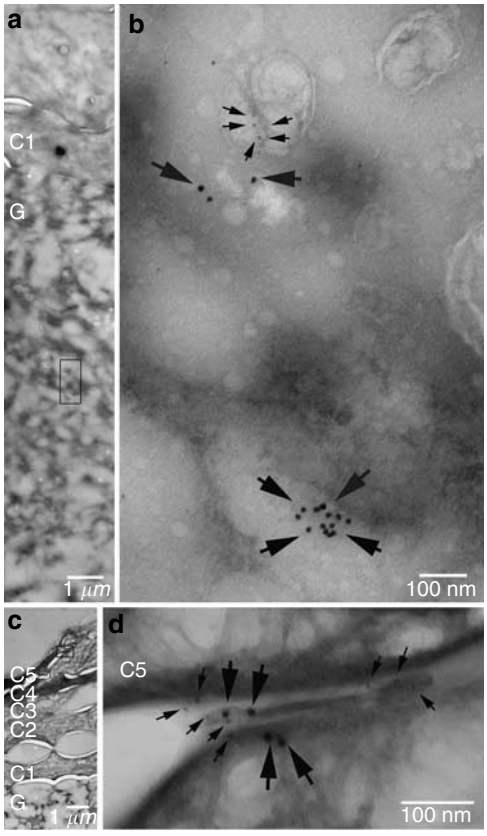

Figure 6. Cystatin M/E and CTSV are colocalized at the desmosomes after secretion. Immunoelectron microscopy using the cryo-ultramicrotomy method. (**a**) Granular layer (G) and the cornified layer (C1). The marked rectangular area is shown at higher magnification in (**b**). Cystatin M/E labels (10 nm gold, lager arrows) are aggregated and not mixed with CTSV labels (5 nm gold, smaller arrows) in the LGs of the granular layer. (**c**) Granular layer and the 1st to 5th cornified layer. (**d**) Higher magnification of the area marked in (**c**), where cystatin M/E and CTSV labels are colocalized at desmosomes.

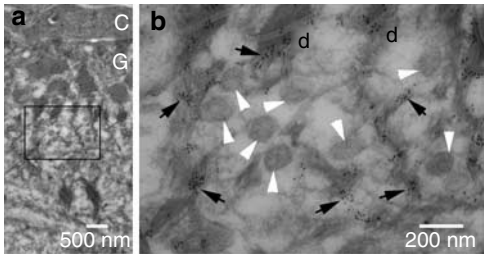

Figure 7. AEP is not an LG protein. Post-embedding immunoelectron microscopy using Lowicryl HM20 resin. (**a**) A lower magnification view of the SC and SG. The rectangular area is shown at higher magnification in (**b**), which shows AEP labels (10 nm gold, black arrows) are not associated with LGs (white arrowheads) but with keratin filaments. d, desmosomes.

M/E-deficient mice (as described in the Introduction) as well CTSL-deficient mice show a skin/hair phenotype (Roth *et al.*, 2000; Benavides *et al.*, 2002). Recently, the phenotype of CTSL knockout mice was rescued by transgenic epidermal re-expression of either murine CTSL or human CTSV, indicating

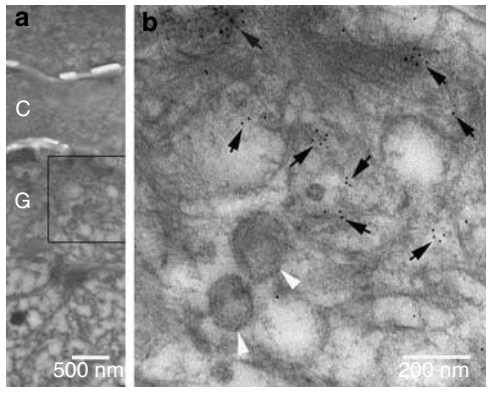

Figure 8. CTSL is not an LG protein. Post-embedding immunoelectron microscopy using Lowicryl HM20 resin. (**a**) A lower magnification view of the SC and SG. The rectangular area is shown at higher magnification in (**b**), which shows CTSL labels (5 nm gold, black arrows) are not associated with LGs (white arrowheads).

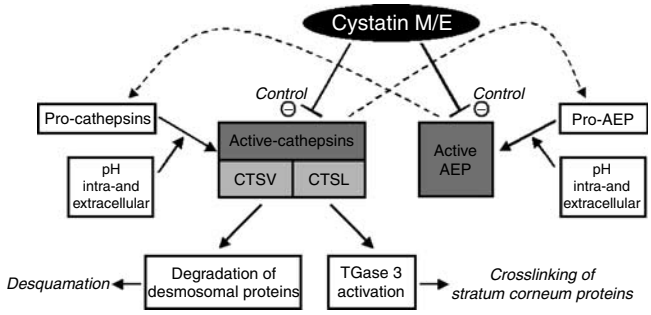

Figure 9. Regulation of epidermal protease activity by cystatin M/E. A simplified scheme of the presumed regulatory role of cystatin M/E in processes that control epidermal cornification and desquamation. Cystatin M/E is an inhibitor of AEP and the cysteine proteases CTSL and CTSV. Inhibition of CTSV activity could possibly regulate desquamation, as CTSV is able to degrade (corneo)-desmosomal proteins. Inhibition of CTSL activity by cystatin M/E is presumably important in the cornification process, as CTSL is the elusive processing and activating enzyme for TGase 3. Inhibition of AEP might regulate the processing of (pro)-cathepsins, a process that is also under control of intra- and extracellular pH changes.

that human CTSV can compensate for murine CTSL (Hagemann *et al.*, 2004; Reinheckel *et al.*, 2005).

We conclude that cystatin M/E has probably two important regulatory functions in epidermal differentiation and hair follicle morphogenesis. First, cystatin M/E could have a role in desquamation by the regulation of CTSV protease activity, which is involved in the degradation of corneodesmosomal components. Second, cystatin M/E could regulate cross-linking of structural proteins by TGase 3 in the cornification process of the epidermis and the hair follicle by controlling CTSL and AEP activities. We have recently developed a human reconstructed skin model that recapitulates the expression pattern of several of the proposed players in this process (unpublished results), and as such will be a valuable tool in further delineation of the role of cystatin M/E and its target proteases in the final steps of epidermal differentiation.

Together with the use of knockout mouse models that we are currently generating (cystatin M/E – AEP and cystatin M/E – CTSL double-knockout mice), this work extends our knowledge on the components that are involved in epidermal cornification and desquamation. We expect that these data will lead to further understanding of human disorders with disturbed skin barrier function.

MATERIALS AND METHODS

Antibodies

Primary antibodies used in this study were as follows: affinity-purified polyclonal rabbit anti-human cystatin M/E antibodies (Zeeuwen *et al.*, 2001), affinity-purified polyclonal sheep anti-human AEP antibodies (Li *et al.*, 2003), monoclonal mouse anti-human CTSV, monoclonal rat anti-human/mouse CTSL, monoclonal mouse anti-human cystatin M/E (all from R&D Systems, Minneapolis, MN), and mouse anti-DSG1-P23 (Progen, Heidelberg, Germany).

For immunofluorescence analysis, the following secondary reagents were used: Alexa-Fluor 488 goat anti-rabbit IgG highly cross-absorbed, and Alexa-Fluor 594 goat anti-rat, goat anti-mouse, and donkey anti-sheep IgG highly cross-absorbed (Molecular Probes, Eugene, OR). Secondary antibodies used for immunoelectron microscopy were 5 or 10 nm gold-conjugated goat anti-mouse IgG, gold-conjugated rabbit anti-goat IgG, and gold-conjugated goat anti-rabbit IgG (all from Amersham Bioscience, Buckinghamshire, UK), and 10 nm gold-conjugated donkey anti-sheep IgG (BBInternational, Cardiff, UK).

Immunofluorescence analysis

Biopsies of normal skin from healthy volunteers were rinsed in phosphate-buffered saline, fixed for 4 hours in buffered 4% formalin, and embedded in paraffin wax (skin biopsies were collected with informed consent using protocols approved by the local medical ethics committee that complies with the Declaration of Helsinki Principles). All material was cut in 7-μm sections, mounted on SuperFrost slides (Menzel, Braunschweig, Germany), deparaffinized, and rehydrated. The sections were subsequently incubated with primary antibodies diluted in 1% BSA/phosphate-buffered saline for 1 hour at room temperature. After washing in phosphate-buffered saline, fluorescent secondary antibodies were applied for 30 minutes at room temperature. For double labeling with antibodies raised in different animals, a mixture of primary antibodies was applied and this was followed by incubation with a mixture of secondary antibodies conjugated with different fluorescent dyes. Nuclei were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DakoCytomation, Copenhagen, Denmark), and the slides were mounted using Prolong Gold Antifade reagent (Molecular Probes). The slides were subsequently examined using an immunofluorescence microscope (Axioskop 2 MOT, Zeiss, Sliedrecht, The Netherlands), and images were photographed using a digital camera (Sony DXC-390P 3 CCD, Scanalytics, Fairfax, VA) and edited using Axiovision Software (Zeiss).

Immunoelectron microscopy

Post-embedding immunoelectron microscopy using Lowicryl K11M resin and Lowicryl HM20 resin (Chemische Werke Lowi, Waldkraiburg, Germany) was performed as described previously (Ishida-Yamamoto *et al.*, 2004). Immunoelectron microscopy on ultrathin cryosections was carried out according to the Tokuyasu method (Tokuyasu, 1989), with slight modifications as described previously (Ishida-Yamamoto *et al.*, 2004).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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